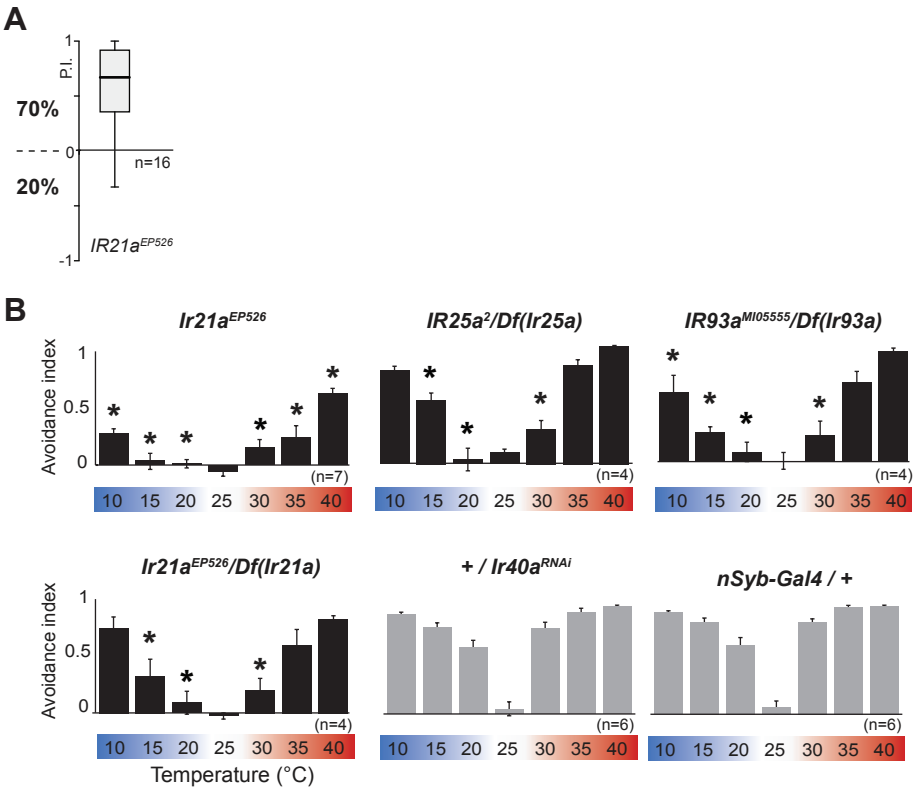

Figure S1, related to Figure 1.

(A) Humidity preference index of *IR21a*^{EP526} flies.

(B) Temperature avoidance index of *IR21a*^{EP526}, *IR21a*^{EP526}*/Df(Ir21a), IR25a*^{2}*/Df(Ir25a), IR93a*^{MI05555}*/Df(Ir93a),* +/*Ir40a*^{RNAi} and *nSyb-Gal4/+* flies.

Temperature avoidance index values were compared by ANOVA, asterisks denote a significant difference between the mutant and genetic background control (*w*$^{1118\ CS}$), P < 0.01; The *nSyb>IR40a*^{RNAi} scores shown in Figure 1 were compared by two-way ANOVA with the relevant *nSyb-Gal4/+* and *UAS-Ir40a*^{RNAi}*/+* controls (shown here in grey), demonstrating no significant interaction; error bars indicate mean ± SEM

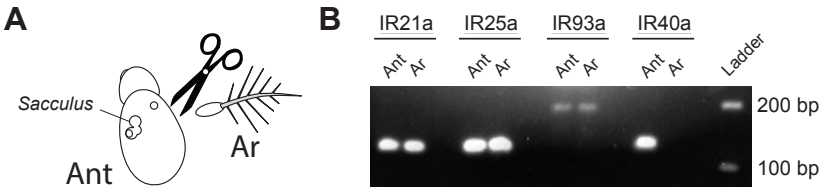

Figure S2, related to Figure 2.
(A) Schematic representation of sample composition (Ant=antenna, Ar=arista).
(B) RT-PCR results divided by gene and sample.

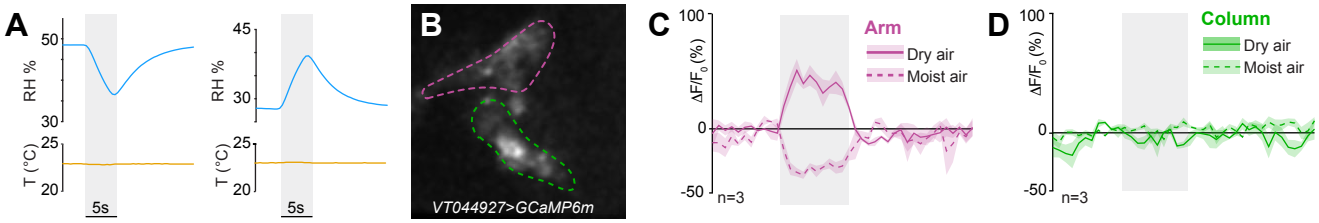

Figure S3, related to Figure 3.
(A) The dry and humid stimulus. The humidity stimulus is ramped, generating a gradual increase, or decrease in RH%.
(B) Single optical plane micrograph from antennal lobe of an *VT044927>GCaMP6m* fly.
(C, D) Averaged traces from (C) Arm and (D) Column of *VT044927>GCaMP6m* flies stimulated with dry or humid air. Shaded areas represent SEM.

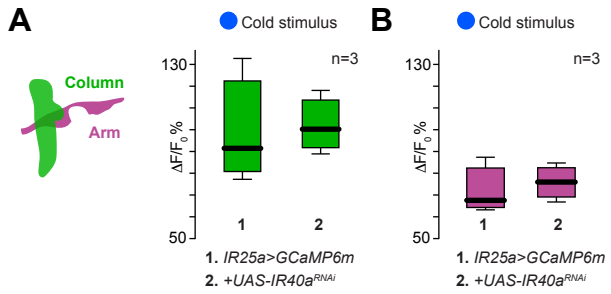

Figure S4, related to Figure 4.
(A) Box plots of median peak values in Column of *IR25a>GCaMP6m* flies exposed to cold stimulus (~3°C)
(B) Box plots of median peak values in Arm of *IR25a>GCaMP6m* flies exposed to cold stimulus (~8°C)

Supplemental Experimental Procedures

Fly Strains

Flies were reared on cornmeal agar medium in room temperature (24°C). For calcium imaging and CaMPARI experiments flies were used 2-5 days post-eclosion. For behavioral experiments flies were 5-15 days old. Only male flies were used in all experiments. Flies were obtained from Bloomington Drosophila Stock Center (BDSC), Vienna Drosophila Resource center (VDRC) or Drosophila Species Stock Center (DSSC) The following flies were used: Canton-special, $w^{1118\ CS}$ (w^{1118} backcrossed to Canton-S ten generations kindly provided by Anne Simon, were used as control), *D. Teissieri* (DSSC 14021-0257.00), *D. mojavensis* (DSSC 15081-1352.32), $wtrw^{1}$ (BDSC 59039), nan^{36a} (BDSC 24902), $Orco^{2}$ (BDSC 23130), $IR8a^{1}$ (BDSC 41744), $IR25a^{2}$ (BDSC 41737), *Df(IR25a)* (BDSC 7496), $IR21a^{EP526}$ (BDSC 17177), *Df(IR21a)* (BDSC 24626), $IR93a^{MI05555}$ (BDSC 42090), *Df(IR93a)* (BDSC 27580), *10XUAS-IVS-mCD8::GFP* (BDSC 32186*), UAS-CaMPARI*^{V398D} (BDSC 58762), *20XUAS-IVS-GCaMP6m* (BDSC 42748 and 42750), *IR25a-Gal4* (BDSC 41728), *IR40a-Gal4* (BDSC 41727), *nSyb-Gal4 2-1* (gift from Julie Simpson), *VT044927-Gal4* (VDRC 207801), *Ir93a-LexA* [19], *UAS-Kir2.1* [S1] and $UAS\text{-}IR40a^{RNAi}$ (VDRC 101725). The following lines were backcrossed to $w^{1118\ CS}$ for 5 generations or more: $Orco^{2}$, $IR8a^{1}$, $IR25a^{2}$, *IR21a*, *IR40a-Gal4, IR25a-Gal4, nSyb-Gal4* and $UAS\text{-}IR40a^{RNAi}$. The UAS-IR93a line was generated by custom synthesis of the full length ORF of IR93a (RefSeq NM_142667.3) that was cloned into the pUAST vector (Eurofins Genomics). Transgenic flies were produced by Bestgene Inc.

Immunohistochemistry, microscopy and image analysis

Antibody staining was performed as previously described with minor modifications [S2]. Brains and antennae were mounted in Rapiclear 1.47 (SunJin Lab). Cuticular autofluorescence was excited with a 561 nm laser, emitted light captured with a 575 nm longpass filter [S3]. The following antibodies were used in this study: rabbit anti-GFP (1:1000, A11122 Life Technologies), mouse anti-nc82 (DSHB, 1:25), goat anti-rabbit Alexa 488 (1:500, A11070 Life Technologies), goat anti-mouse Alexa 546 (1:500, A11030 Life Technologies). Imaging was performed on a Zeiss LSM 510 META confocal microscope with a 20x or 40x objective at a resolution of 1024x1024. Transmission electron microscopy was performed essentially as described [S4]. Micrographs were captured at 80 kV on a JEOL 1230 fitted with a Gatan 794 CCD multiscan camera. Images were processed in ImageJ 1.49v (NIH). Live imaging of GFP-labeled projections to the PAL (**Figure 3A-D**) was performed on a Prairie Ultima two-photon microscopy system equipped with a GaAsP PMT, and an Olympus 40× water immersion objective at a resolution of 512x512 and 2.5x optical zoom. Maximum projections of the PAL were obtained from stacks taken at 1µm steps and processed in Fiji [S5].

RT-PCR

Total RNA from approximately 200 aristae or 20 antennae (comprising segments 2 and 3, but lacking the arista) was extracted using standard methods (TRIzol, Invitrogen). cDNA library was obtained with a cDNA iScript Select cDNA Synthesis Kit (BioRad). Gene-specific primers for RT-PCR were designed to flank an intron and their sequences were as following: Ir21a RT F (5'- GCAACGTTACCCAGAGCTTC-3') and Ir21a RT R (5'-CTCAAATGAAGCGCCGATCG-3'); Ir25a RT F (5'-GCAGGGATCGCATCTCAAGGATCAG-3') and Ir25a RT R (5'-GCTTATCGCACTTGGCCAGAGCTTC-3'); Ir40 RT F (5'- CTCAAACCCAGAACTGTGCTGTCTG-3') and Ir40 RT R (5'- GGCTCGGTATCCGTTATGTAGTTC-3'); Ir93a RT F (5'- CTGTACGCCATTAATCGCCTAGCTC-3') and Ir93a RT R (5'- GTCACCAGCACGATAACCACGATC-3').

Calcium imaging

Calcium imaging of humidity [S2] and temperature stimuli [19] was performed essentially as previously described. To generate dry and humid stimuli, headspace from vials with saturated solutions of LiCl (dry) and NaCl (humid) were used. DEET (Sigma-Aldrich) was dissolved in DMSO. DMSO was used as baseline stimulus. Images were acquired at 256 x 256 pixel resolution at a rate of 2 Hz on a Prairie Technologies two-photon microscope using an Olympus 40x 0.9 NA objective. A MaiTai laser (Spectra-Physics) tuned to 925 nm was used for excitation. Fluorescence was collected with GaAsP photomultiplier tubes (Hamamatsu). Analysis of fluorescence intensity dynamics was performed in ImageJ using the measure stack function of a circular region of interest 15 pixels in diameter, after image correction was performed using the StackReg function (**Figure 3**). To calculate change in fluorescence we used the formula $\Delta F/F_0 = (F_t - F_0)/F_0$, where F_0 is the baseline fluorescence determined by averaging 5 frames before stimulus onset and F_t is the mean fluorescent value at a given time. Imaging and data analysis shown in Figure 4 were performed as described in detail in [19].

Campari

For CaMPARI experiments, flies were mounted as for Calcium imaging except that head cuticle was left intact. Headspace from vials containing saturated LiCl or NaCl was used as dry and humid stimulus. Simultaneously as a 5 second stimulus was delivered, a 500 ms flash of 400 nm light was delivered at 1 Hz to the head of the fly, focused through a 10x objective. This was repeated with 20 seconds delay between trials until 200 seconds of photoconversion was achieved. Brain were then dissected and mounted in PBS with 5 mM EGTA and immediately imaged on a confocal microscope. To image the green channel, we included a low-power 405 nm laser line together with the 488 nm laser line to reduce photoswitching, as described [21]. For image analysis in ImageJ, we used the remove noise function for the red channel (to remove specks) and a Gaussian blur filter (radius 1) on both channels. To calculate change in fluorescence we used the formula $\Delta F/F_{\text{green}} = (F_{red} - F_{\text{green}})/F_{\text{green}}$.

Behavioral experiments

Humidity preference assay: In a 48-well plate, wells were filled with a saturated solution of LiCl, NaCl or KH_2PO_4 (Sigma-Aldrich). The wells were covered with a polyamide mesh (Sintab Produkt AB) and a polyamide 3d-printed custom frame (i.materialize) was placed on the net to create six lanes. Flies were cold anesthetized before the experiment and then distributed 5-8 flies per lane and covered with a lid. The plates were placed on top of transparent plastic boxes (15 cm high) to avoid heating from the light source (Artograph LightPad A940). Movies were captured with a webcam (Logitech C930e) controlled using a custom script written in Matlab (Mathworks). Flies were manually scored and a preference index was calculated PI = (# flies on moister sider - #flies on drier sider)/total # flies. Humidity was measured using an EK-H4 multiplexer equipped with SHT71 sensors and recorded with the EK-H4 viewer software (Sensirion). Temperature preference assay were performed essentially as described [19]. Briefly, 15-20 flies are placed into small chambers whose floor is tiled by four independently-controlled Peltier elements. The temperature of each tile is set via custom software, and over the course of each experiment, a defined sequence of temperature pairs (TT°C, or "test" temperature, vs. the preferred 25°C) is sent to each chamber for 3 minutes (presented to the flies in two different but symmetrical spatial configurations to account for potential bias in distribution). Finally, an Avoidance Index (AI) for each TT is calculated by plotting the flies' positions within the chamber (recorded at 3Hz with a CCD camera) frame-by-frame as follows: (AI) for each TT: AI=# flies at 25 °C − # flies at TT/(total # flies). AI values were compared using ANOVA or 2-way ANOVA as previously described [19]. Avoidance index values for experiments with Gal4 and UAS lines were compared by two-way ANOVA (threshold $P = 0.01$). Kolmogorov–Smirnov tests were used to confirm a normally distributed sample. Homogeneity of variance for each data set was confirmed by calculating the Spearman correlation (ρ) between the absolute values of the residual errors and the observed values of the dependent variable (threshold $P = 0.05$). Statistical analysis was carried out in MATLAB; sample sizes were chosen to reliably measure experimental parameters. Experiments did not involve randomization or blinding. All temperature preference experiments were performed in a custom chamber kept at a constant RH of 40%.

Supplemental References

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